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TENT COOPERATION TRE. Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 February 2001 (12.02.01)	
International application No. PCT/PT00/00007	**Applicant's or agent's file reference**
International filing date (day/month/year) 09 June 2000 (09.06.00)	**Priority date** (day/month/year) 09 June 1999 (09.06.99)
Applicant SOARES PAIS, Maria, Salomé et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

08 January 2001 (08.01.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

Telephone No.: (41-22) 338.83.38

ATENT COOPERATION TRE[redacted]Y

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
...		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/PT00/00007	09/06/2000	09/06/1999
International Patent Classification (IPC) or national classification and IPC		
C12N1/19		
Applicant		
INSTITUTO DE CIENCIA APLICADA E... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
08/01/2001	08.11.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Halle, F Telephone No. +49 89 2399 8537

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/PT00/00007

I. Basis f th report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-6	as received on	01/10/2001	with letter of	28/09/2001

Claims, No.:

1-21	as received on	01/10/2001	with letter of	28/09/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-21
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-21
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-21
	No:	Claims	

2. Citations and explanations
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/PT00/00007

Point V

1. In this report, it is referred to the following documents:

   D1: Biochimica et Biophysica Acta 1206, 1994, p. 279-285
   D2: EP 0 123 289

2.1 Having regard to the prior art D1 and D2, the subject-matter of claims 1-21 appears to be novel (Article 33(2) PCT), since, in particular, the prior art does not disclose the production of a plant aspartic proteinase by using yeast as a host cell.

2.2 Concerning inventive step, it is agreed with the Applicant stating that the efficient combination of the pre and pro sequence fused to the mature part of the plant gene in such a way that an active protein is produced results in an accurate method which is not evident for a skilled person; this efficient combination is based on technical features which appear to be essential to the invention. However, these features are not mentioned in the "method" claim 1. Therefore, the subject-matter of all the claims 1-21 would be considered as inventive (Article 33(3) PCT), provided that the "method" claim 1 also explicitly refers to the above mentioned technical features.

3. The end of the text of claim 11 seems to be incomplete.

4. In the independent claim 19, the antibody should also be characterized as anti-plant aspartic proteinase polyclonal antibody since the designation CCMP1 alone in the claim is an internal designation without signification to the public and thus renders the independent claim unclear.

DESCRIPTION

Production by yeasts of aspartic proteinases from plant origin with sheep's, cow's, goat's milk, etc. clotting and proteolytic activity.

BACKGROUND OF THE INVENTION

Field of the Invention

The use of a yeast expression system has become a way of producing large quantities of different types of compounds on an industrial scale. Regarding the production of plant-origin aspartic acid proteinases with industrial applications, there has not been any news of yeast expression with regard to production for use on an industrial scale.

The object of this invention patent, described below, refers to the construction of plasmids, the transformation of yeast strains and the production of plant-origin aspartic acid proteinases. These proteins are proteolytic and milk clotting enzymes which can be used in the cheese production and other biotechnological applications

Description of the Prior Art

Plant aspartic proteinases have been isolated, characterised and cDNA have been prepared since 1997 (D'Hondt et al, 1997). The studies with the aspartic proteinases derived from *Cynara cardunculus* named Cyprosins started in the nineties, with the purification of the enzymes (at that time known as Cynarases, Heimgartner *et al*, 1990), followed in 1992 with their partial characterisation. The construction of a cDNA library and the isolation of a cDNA clone was first reported in 1993 (Cordeiro 1993) and published in several journals since 1994, together with the characterisation of their tissue specificity (Brodelius *et al*, 1995; Cordeiro *et al*, 1994; 1994; Cordeiro *et al*, 1995). The sequence of the CYPRO11 cDNA was included in the gene bank and reported later on (Brodelius *et al*, 1998). Purification of Cardosins, the other group of *Cynara cardunculus* aspartic proteinases, was achieved in 1995 (Faro *et al*, 1995). After this, an extensive work was performed with respect to some biochemical properties including specificity towards substrates (Faro *et al*, 1995; Veríssimo *et al*,1995, 1996). Characterisation and partial protein sequence analysis started in 1995 (Faro *et al*, 1995; Veríssimo *et al*, 1996). Since then, the studies performed in further characterisation of the enzymes, their glycosylation pattern (Costa *et al*, 1997), their

histological and cytological location (Ramalho-Santos *et al*, 1997) and function (Faro *et al*, 1998) have been published. The characterisation of the enzyme precursor (Ramalho-Santos *et al*, 1998a) and identification of its proteolytic processing mechanism (Ramalho-Santos *et al*, 1998b) helped to understand the molecular and physiological relevance of the intra-molecular domains such as the pro-sequence and the plant-specific-insert. Crystallisation studies on the structure of Cardosin A started in 1998 (Bento *et al*, 1998) and has contributed to the knowledge of intramolecular aspects related to the biological function (Frazão *et al*, 1999). Only very recently the cDNA encoding the Cardosin A was cloned. Functional aspects of protein domains and motifs and further implications in the function of this enzyme were better clarified (Faro *et al*, 1999).

The description of a DNA construct for expression of polypeptides by yeast cells was prior reported (EP 0123289). The constructs employed the entire yeast α-factor secretion leader. Since then the production of several polypeptides of interest have been reported in yeast cells, including aspartic proteinases from animal origin, as for example bovine chymosin (Mellor et al, Gene 1983, 24: 1-14), and human cathepsin E (Yamada et al, Biochimica et Biophysica Acta 1994, 1206: 279-285).

EXPERIMENTAL

Construction of Plasmids. Transformation of Yeast Strains and Production of Plant Aspartic Proteinases

The insertion of coding gene CYPRO11 into a plant-origin proteinase constitutes the experimental model for controlling the yeast expression of plant-origin aspartic acid enzymes.

Two *Escherichia coli*-yeast expression system vectors were constructed, using a type 2μ multi-copy plasmid and a centromeric plasmid having a low number of copies. The choice of gene used was the leucine deficient one (LEU2). The expression cassette contained developer Gal7 promotor and four different leader sequences upstream from the heterologous gene. Transcription of the heterologous gene was stopped by a PGK1 terminator.

From the different leader sequences tested (native prosequence, preSUC2-proCYPRO11, preMFα-proCYPRO11 and preproMFα), we concluded that preMFα-proCYPRO11 was the best leader sequence for the production of plant-origin aspartic acid proteinases, whether cyprosins corresponding to the plant-origin model proteins coded by gene CYPRO11, or other commercially interesting plant-origin acidic aspartic proteinases.

The MFα yeast presequence is sufficient to develop secretion of the aspartic acid proteinase into the culture medium, and the use of a prosequence of the gene is not necessary. The native prosequence was essential to the active protein's production.

The use of centromeric plasmids having a low number of copies gave better results than type 2μ multi-copy plasmids.

Different yeast strains were tested, including *Saccharomyces cerevisae* BJ1991 (MATα *leu2 trpl ura3-52 prbl-1122 pep4-3*), BJ2168 (MATα *leu2 trpl ura3-52 prcl-1122 pep4-3*), MT302/1c-a (*arg5-6 leu2-12 his3-11 his3-15 peb4-3 ade1*), W303-1^a (*MATα leu2-3,112 ura3-1 trpl-1 his3-11,15 ade2-1 can1-100* GAL SUC2).

These strains were kept on YPD agar plates containing 1% yeast extract, 2% bacto-peptone, 2% glucose and 1.5% agar.

The transformed yeast was grown in an SD medium (0.67% yeast nitrogen base without amino acids, DIFCO, 2% (w/v) glucose), supplemented with amino acids suited to the auxotrophic needs of each strain, except for the leucine one.

The cultures were collected and washed once with sterile distilled water. The cells were resuspended in a YPGal medium (1% yeast extract, 2% bacto-peptone, 4% galactose) and used to inoculate the same medium at a density of A_{600} = 0.2. The cultures were incubated in the same culture conditions until they reached densities of A_{600} = 2, 6 or 10.

Of the yeast strains tested, protease deficient strain BJ1991 produced and secreted into the culture medium the largest quantities of aspartic acid proteinase with considerable milk clotting and proteolytic activity. The secretion of proteolytic enzymes was therefore dependent on culture growth. The recombinant proteinase

with the highest degree of clotting and proteolytic activity was obtained in the stationary phase of the YPGal medium's growth (A_{600} = 10). In the exponential phase (A_{600} = 2), the yeast cells secreted an inactive recombinant proteinase having a high molecular weight. It was considered to be an unprocessed form of the proteinase in which a specific region of the genes of plant-origin acidic aspartic proteinases called a specific plant insert had not been removed.

The largest sub-unit of the recombinant proteinases secreted by the yeast was glycosilated, in the only site possible for glycosilation, and contained a considerable number of manose type glycan chains.

Preparation of Polyclonal Antibodies

The total proteic extract used to produce polyclonal antibodies against plant-origin acidic aspartic proteinase with considerable coagulation and proteolytic activity was obtained from the dry flowers of *Cynara cardunculus* by maceration in a mortar in liquid nitrogen and extraction with 50mM of Tris HCl buffer at a pH of 8.3 (Heimgartner et al., 1990). The proteins were fractionated in 12% SDS-PAGE using 100µg of total protein extract per well. The gel was tinted with a 0.02% Commassie Blue solution in distilled water. The bands corresponding to the largest sub-unit of the plant enzyme (31-32.5kDa in the SDS-PAGE gel) were isolated and the content of each well was sent to EUROGENTEC (Belgium) for the production of antibodies.

Isolation of the Plant-origin Proteinase and Western Blotting Analysis

Isolation of the recombinant plant-origin proteinase from the cell extracts was done using 30ml of yeast cells grown to densities of A_{600} = 2, 6 or 10. After collection, the cells were washed with distilled water, resuspended in 500µl of buffer and exploded by shaking them with glass balls.

Isolation of the recombinant proteinase from the culture medium was done after collecting the medium and concentrating it almost 10 times by ultracentrifugation.

The proteinase concentration was ascertained using the Bio-Rad protein analysis kit in accordance with the manufacturer's instructions. 50µg of total proteic extract from the yeast cells or 1.125g of the concentrated culture medium was analysed in 12%

SDS-PAGE. The proteins were transferred to a nitro-cellulose membrane (Bio-Rad) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) equipment in accordance with the manufacturer's instructions. Proteins were detected using polyclonal antibody CCMPI prepared in accordance with the description in the previous section and Boeringer Mannheim's Chemiluminescence Western Blotting Kit in accordance with the manufacturer's instructions.

The results obtained showed that the transformed yeast produces plant-origin aspartic acid proteinase and that the inactive form is found in cells in the exponential growth phase while the active form is secreted into the culture medium. This peculiarity is decisive when it comes to getting good performance for the extraction and purification of plant-origin acidic aspartic proteinases produced from yeast.

Analysis of the Plant-origin Recombinant Enzyme's Clotting and Proteolytic Activity

Proteolytic activity was analysed in accordance with the Twinning method (1984). The casein preparation marked with isothiocyanate (casein-FTC) was made in accordance with the author's instructions. The reactive mixture contained 30µl of 0.2M sodium citrate buffer, pH 5.1, 20l of casein-FTC and 20µl of enzyme solution (3µg/µl in the case of total proteic extract from the yeast cells or 150ng/µl in the case of concentrated culture medium).

Two control tests were done by replacing the enzymatic solution with the reactive buffer. Another control was performed by using the same yeast strain transformed with the same plasmids in which the heterologous gene was absent. The samples were incubated at 37°C for 30 minutes. Reaction was stopped by adding 120µl of 5% trichloracetate acid (TCA) in all but one of the controls. In the latter case, the same amount of 0.5M Tris HCI buffer at a pH of 8.0 (positive control) was added. The samples were centrifuged and a 150µl aliquot of the supernatant fraction was diluted to 3ml with 0.5M Tris HCI buffer at a pH of 8.5. The control (without enzymes), whose reaction was stopped with the TCA solution, was used to ascertain the formation of soluble fluorescent compounds in TCA with enzyme intervention. Relative fluorescence of the samples was ascertained using wavelengths of 490nm for excitation and 525nm for emission in a Shimadzu RF-1501 (Shimadzu Corporation,

Kyoto, Japan) spectrofluorimeter. The percentage of relative fluorescence (%RF) was calculated by subtracting the negative control values from the values, and considering the positive control values as 100%RF. For statistical analysis of the results, each sample had three replicas and three independent readings were taken. The data obtained were analysed with the Student's *t* test (α=0.05). Greatest proteolytic activity, obtained for the best combination/yeast strain, was 15% RF/μ of protein. This figure refers to standard culture conditions, and can be increased under conditions optimised for industrial purposes namely using mutant yeast strains chosen for their maximum recombinant proteinase secretion into the culture medium.

Ascertaining Clotting Activity

Clotting activity was ascertained in test tubes, using unconcentrated culture medium in accordance with the following method: 10ml of the culture medium of the transformed YPGal yeast cells was added to 3ml 12% of skimmed milk (bacto-Difco) and 100ml mM $CaCl_2$. The pH of the culture medium for the culture grown to either A_{600} = 6 or 10 was approximately 5.0. For the culture medium of the culture grown to A_{600} = 2, the pH was adjusted to 5.0 using HCl. The samples were kept at 37°C until the onset of coagulation. The coagulation was evident.

CLAIMS

1. A method for producing an aspartic proteinase from plant origin using yeast as a host cell said method comprising the introducing into that host cell a DNA construct containing the sequence encoding the said aspartic proteinase from plant origin and growing said host cell comprising said DNA construct containing the sequence in a culture medium whereby said aspartic proteinase from plant origin or part thereof is secreted or not into the culture medium

2. A method according to Claim 1 whereby said DNA sequence forms part of a DNA construct which is introduced into said host cell and which comprises in the direction of transcription a pro sequence heterologous to said host cell or to said aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the said DNA sequence coding for the mature aspartic proteinase from plant origin whereby said aspartic proteinase from plant origin is secreted by said host cell

3. A method according any one of Claims 1 and 2 wherein said aspartic proteinase from plant origin is a plant enzyme

4. A method according to 1 to Claim 3, wherein said enzyme is a plant aspartic proteinase or an unprocessed form thereof

5. A method according to Claim 1 to 4 wherein said enzyme is cyprosin or mutant forms thereof

6. A method according to any one of the Claims 1 to 4 wherein said aspartic proteinase from plant origin is cardosin or mutant forms thereof

7. A method according to any one of Claims 1 to 6 wherein said host cell is an yeast strain with laboratory or industrial interest

8. A method according to any one of Claims 1 to 7 wherein said host cell is from the genus *Saccharomyces* used for the transformation and expression of plant aspartic proteinases encoding genes and the secretion of the aspartic proteinase from plant origin encoded by said genes or secretion of part of said aspartic proteinase from plant origin

9. A transformed yeast host cell comprising an expression cassette which comprises, in the direction of transcription a leader sequence functional in said host cell composed of a pro-sequence heterologous to said host cell or to an aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the DNA sequence encoding for the said mature aspartic proteinase from plant origin

10. A cell according to Claim 9 wherein said pro-sequence is a plant aspartic proteinase pro-sequence

11. A cell according to Claims 9 and 10 wherein said aspartic proteinase is a plant aspartic proteinase or a thereof

12. A cell according to Claim 9, 10 and 11 wherein said aspartic proteinase from plant origin is cyprosin or an unprocessed form thereof

13. A cell according to Claim 9, 10, 11 and 12 wherein said aspartic proteinase from plant origin is cardosin or an unprocessed form thereof

14. The expression cassettes constructs for use in a yeast host cells comprising: in the direction of transcription a leader sequence composed of a pro-sequence heterologous to said host cell or to aspartic proteinase from plant origin and said pro-sequence is joined in reading frame to the DNA sequence encoding for the said mature aspartic proteinase from plant origin

15. The expression cassettes constructs according to Claim 14, wherein said pro-sequence is heterologous to said host cell or to said aspartic proteinase from plant origin or to said host cell and said aspartic proteinase from plant origin

16. The expression cassettes constructs according to Claim 14 or 15 further comprising the pro-sequence of the plant aspartic proteinase and the plant gene encoding plant aspartic proteinases

17. A method according to any one of Claims 1 to 8 wherein said aspartic proteinase from plant origin or part thereof is isolated either from the cell extracts or from the culture medium

18. A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the antibody raised against the said aspartic proteinase from plant origin

19. A method for detection of the aspartic proteinase from plant origin either in the cell extracts or in the culture medium using the antibody CCMP1

20. The transformed yeast cells in culture described in Claims 9 to 13 characterised by their production of recombinant plant aspartic proteinases with milk clotting activity which cleave caseins from milk of different origins, namely sheep's, cow's and goat's milk confirmed by milk clotting tests

21. The transformed yeast cells in culture described in Claims 9 to 13 characterised by their production of recombinant plant aspartic proteinases including cyprosins and cardosins capable of giving to cheese a special taste and flavour

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/PT 00/ 00007	09/06/2000	09/06/1999
Applicant		
INSTITUTO DE CIENCIA APLICADA E TECNOLOGIA (ICAT)		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PT 00/00007

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/19 //C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CORDEIRO MARIA C ET AL: "Isolation and characterization of a cDNA from flowers of Cynara cardunculus encoding cyprosin (an aspartic proteinase) and its use to study the organ-specific expression of cyprosin." PLANT MOLECULAR BIOLOGY, vol. 24, no. 5, 1994, pages 733-741, XP002149832 ISSN: 0167-4412 the whole document --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2000	27/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]PT 00/00007

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	D'HONDT KATHLEEN ET AL: "Aspartic proteinase genes in the Brassicaceae Arabidopsis thaliana and Brassica napus." PLANT MOLECULAR BIOLOGY, vol. 33, no. 1, 1997, pages 187-192, XP002149835 ISSN: 0167-4412 the whole document	1-7
Y	YAMADA MASAYUKI ET AL: "Secretion of human intracellular aspartic proteinase cathepsin E expressed in the methylotrophic yeast, Pichia pastoris and characterization of produced recombinant cathepsin E." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1206, no. 2, 1994, pages 279-285, XP000952761 ISSN: 0006-3002 the whole document	1-7
Y	EP 0 123 289 A (CHIRON CORP) 31 October 1984 (1984-10-31) the whole document	1-7
P,X	WHITE PAUL C ET AL: "Processing, activity, and inhibition of recombinant cyprosin, an aspartic proteinase from Cardoon (Cynara cardunculus)." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 24, 11 June 1999 (1999-06-11), pages 16685-16693, XP002149833 ISSN: 0021-9258 the whole document	1-7

1

INTERNATIONAL SEARCH REPORT

Inf n on patent family members

International Application No
[redacted]/PT 00/00007

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0123289 A	31-10-1984	US 4588684 A	13-05-1986
		CA 1240940 A	23-08-1988
		DK 92884 A	27-10-1984
		JP 59198980 A	10-11-1984

DESCRIPTION

Production by yeasts of aspartic proteinases from plant origin with sheep's, cow's, goat's milk, etc. clotting and proteolytic activity.

Introduction

The use of a yeast expression system has become a way of producing large quantities of different types of compounds on an industrial scale. Regarding the production of plant-origin aspartic acid proteinases with industrial applications, there has not been any news of yeast expression with regard to production for use on an industrial scale.

The object of this invention patent, described below, refers to the construction of plasmids, the transformations of yeast strains and the production of plant-origin aspartic acid proteinases.

Construction of Plasmids. Transformation of Yeast Strains and Production of Plant-origin Proteinases

The insertion of coding gene CYPRO11 into a plant-origin proteinase constitutes the experimental model for controlling the yeast expression of plant-origin aspartic acid enzymes.

Two *Escherichia coli*-yeast expression system vectors were constructed, using a type 2μ multi-copy plasmid and a centromeric plasmid having a low number of copies. The choice of gene used was the leucine deficient one (LEU2). The expression cassette contained developer G7 and four different leader sequences upstream from the heterologous gene. Transcription of the heterologous gene was stopped by a PGK1 terminator.

From the different leader sequences tested (native prosequence, preSUC2-proCYPRO11, preMFα-proCYPRO11 and preproMFα), we concluded that preMFα-proCYPRO11 was the best leader sequence for the production of plant-origin aspartic acid proteinases, whether cyprosins corresponding to the plant-origin model proteins coded by gene CYPRO11, or other commercially interesting plant-origin acidic aspartic proteinases.

The MFα yeast presequence is sufficient to develop secretion of the aspartic acid proteinase into the culture medium, and the use of a prosequence of the gene is not necessary. The native prosequence was essential to the active protein's production.

The use of centromeric plasmids having a low number of copies gave better results than type 2μ multi-copy plasmids.

Different yeast strains were tested, including *Saccharomyces cerevisae* BJ1991 (MATα *leu2 trpl ura3-52 prbl-1122 pep4-3*), BJ2168 (MATα *leu2 trpl ura3-52 prcl-1122 pep4-3*), MT302/1c-a (*arg5-6 leu2-12 his3-11 his3-15 peb4-3 ade1*), W303-1ª (*MATα leu2-3,112 ura3-1 trpl-1 his3-11,15 ade2-1 can1-100* GAL SUC2).

These strains were kept on YPD agar plates containing 1% yeast extract, 2% bacto-peptone, 2% glucose and 1.5% agar.

The transformed yeast was grown in an SD medium (0.67% yeast nitrogen base without aminoacids, DIFCO, 2% (w/v) glucose), supplemented with aminoacids suited to the auxotrophy needs of each strain, except for the leucine one.

The cultures were collected and washed once with sterile distilled water. The cells were resuspended in a YPGal medium (1% yeast extract, 2% bacto-peptone, 4% galactose) and used to inoculate the same medium at a density of A_{600} = 0.2. The cultures were incubated in the same culture conditions until they reached densities of A_{600} = 2, 6 or 10.

Of the yeast strains tested, protease deficient strain BJ1991 produced and secreted into the culture medium the largest quantities of aspartic acid proteinase with considerable milk clotting and proteolytic activity. The secretion of proteolytic enzymes was therefore dependent on culture growth. The recombinant proteinase with the highest degree of clotting and proteolytic activity was obtained in the stationary phase of the YPGal medium's growth (A_{600} = 10). In the exponential phase (A_{600} = 2), the yeast cells secreted an inactive recombinant proteinase having a high molecular weight. It was considered to be an unprocessed form of the proteinase in which a specific region of the genes of plant-origin acidic aspartic proteinases called a specific plant insert had not been removed.

The largest sub-unit of the recombinant proteinases secreted by the yeast was glycosilated, in the only site possible for glycosilation, and contained a considerable number of manose type glycan chains.

Preparation of Polyclonal Antibodies

The total proteic extract used to produce polyclonal antibodies against plant-origin acidic aspartic proteinase with considerable coagulation and proteolytic activity was obtained from the dry flowers of *Cynara cardunculus* by maceration in a mortar in liquid nitrogen and extraction with 50mM of Tris HCl buffer at a pH of 8.3 (Heimgartner et al., 1990). The proteins were fractionated in 12% SDS-PAGE using 100μg of total protein extract per well. The gel was tinted with a 0.02% Commassie Blue solution

in distilled water. The bands corresponding to the largest sub-unit of the plant enzyme (31-32.5kDa in the SDS-PAGE gel) were isolated and the content of each well was sent to EUROGENTEC (Belgium) for the production of antibodies.

Isolation of the Plant-origin Proteinase and Western Blotting Analysis

Isolation of the recombinant plant-origin proteinase from the cell extracts was done using 30ml of yeast cells grown to densities of A_{600} = 2, 6 or 10. After collection, the cells were washed with distilled water, resuspended in 500µl of buffer and exploded by shaking them with glass balls.

Isolation of the recombinant proteinase from the culture medium was done after collecting the medium and concentrating it almost 10 times by ultracentrifugation.

The proteinase concentration was ascertained using the Bio-Rad protein analysis kit in accordance with the manufacturer's instructions. 50µg of total proteic extract from the yeast cells or 1.125g of the concentrated culture medium was analysed in 12% SDS-PAGE. The proteins were transferred to a nitro-cellulose membrane (Bio-Rad) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) equipment in accordance with the manufacturer's instructions. Proteins were detected using polyclonal antibody CCMPI prepared in accordance with the description in the previous section and Boeringer Mannheim's Chemiluminescence Western Blotting Kit in accordance with the manufacturer's instructions.

The results obtained showed that the transformed yeast produces plant-origin aspartic acid proteinase and that the inactive form is found in cells in the exponential growth phase while the active form is secreted into the culture medium. This peculiarity is decisive when it comes to getting good

performance for the extraction and purification of plant-origin acidic aspartic proteinases produced from yeast.

Analysis of the Plant-origin Recombinant Enzyme's Clotting and Proteolytic Activity

Proteolytic activity was analysed in accordance with the Twinning method (1984). The casein preparation marked with isothiocyanate (casein-FTC) was made in accordance with the author's instructions. The reactive mixture contained 30µl of 0.2M sodium citrate buffer, pH 5.1, 20l of casein-FTC and 20µl of enzyme solution (3µg/µl in the case of total proteic extract from the yeast cells or 150ng/µl in the case of concentrated culture medium).

Two control tests were done by replacing the enzymatic solution with the reactive buffer. The samples were incubated at 37°C for 30 minutes. Reaction was stopped by adding 120µl of 5% trichloracetate acid (TCA) in all but one of the controls. In the latter case, the same amount of 0.5M Tris HCl buffer at a pH of 8.0 (positive control) was added. The samples were centrifuged and a 150µl aliquot of the supernatant fraction was diluted to 3ml with 0.5M Tris HCl buffer at a pH of 8.5. The control (without enzymes), whose reaction was stopped with the TCA solution, was used to ascertain the formation of soluble fluorescent compounds in TCA with enzyme intervention. Relative fluorescence of the samples was ascertained using wavelengths of 490nm for excitation and 525nm for emission in a Shimadzu RF-1501 (Shimadzu Corporation, Kyoto, Japan) spectrofluorimeter. The percentage of relative fluorescence (%RF) was calculated by subtracting the negative control values from the values, and considering the positive control values as 100% RF. For statistical analysis of the results, each sample had three replicas and three independent

readings were taken. The data obtained were analysed with the Student's *t* test (α=0.05). Greatest proteolytic activity, obtained for the best combination/yeast strain, was 15% RF/μ of protein. This figure refers to standard culture conditions, and can be increased under conditions optimised for industrial purposes and using Sec- strains, that is, strains chosen for their maximum recombinant proteinase secretion into the culture medium.

Ascertaining Clloting Activity

Clotting activity was ascertained in test tubes, using unconcentrated culture medium in accordance with the following method: 10ml of the culture medium of the transformed YPGal yeast cells was added to 3ml 12% of skimmed milk (bacto-Difco) and 100ml mM $CaCl_2$. The pH of the culture medium for the culture grown to either A_{600} = 6 or 10 was approximately 5.0. For the culture medium of the culture grown to A_{600} = 2, the pH was adjusted to 5.0 using HCl. The samples were kept at 37°C until the onset of coagulation. The coagulation was evident.

CLAIMS

1. Transformed yeast cultures are characterised by their content in coding genes for plant-origin aspartic acid proteinases and their production of plant-origin aspartic acid proteinases with milk clottting activity, secreted in active form into the culture medium. They can be extracted from the culture medium, purified and supplied in liquid or lyophilised form on a domestic or industrial scale as milk clotting enzymes.

2. The transformed yeast cultures described in claim 1 are characterised by the stable integration of coding genes for plant-origin aspartic acid proteinases.

3. The transformed yeast cultures described in claims 1 and 2 are characterised by their ability to produce aspartic acid proteinases, confirmed by the use of produced antibodies against cardoon aspartic acid proteinases, and their milk clotting activity, confirmed by milk clotting tests.

4. The transformed yeast cultures described in claims 1, 2 and 3 are characterised by their ability to secrete recombinant aspartic acid proteinases into the culture medium.

5. The transformed yeast cultures described in claims 1, 2, 3 and 4 are characterised by their ability to produce recombinant aspartic acid proteinases capable of effectively coagulating milk from different origins, especially sheep's, cow's and goat's milk.

6. The transformed yeast cultures described in claims 1, 2, 3, 4 and 5 are characterised by their ability to produce aspartic acid proteinases which cleave α, β and κ caseins from milk from different origins.

7. The transformed yeast cultures described in claims 1, 2, 3, 4 are characterised by their production of recombinant aspartic acid proteinases, including cyprosins and cardosins, capable of giving cheese a special flavour, smell and consistency.

INTERNATIONAL SEARCH REPORT

Intern[illegible] Application No
PCT/PT 00/00007

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/19 //C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CORDEIRO MARIA C ET AL: "Isolation and characterization of a cDNA from flowers of Cynara cardunculus encoding cyprosin (an aspartic proteinase) and its use to study the organ-specific expression of cyprosin." PLANT MOLECULAR BIOLOGY, vol. 24, no. 5, 1994, pages 733-741, XP002149832 ISSN: 0167-4412 the whole document --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	D'HONDT KATHLEEN ET AL: "Aspartic proteinase genes in the Brassicaceae Arabidopsis thaliana and Brassica napus." PLANT MOLECULAR BIOLOGY, vol. 33, no. 1, 1997, pages 187-192, XP002149835 ISSN: 0167-4412 the whole document	1-7
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Interr... n... ...ication No
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		CA 1240940 A	23-08-1988
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		JP 59198980 A	10-11-1984